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Comprehensive Scanning of the Entire Mitochondrial Genome for Mutations

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Background: **Definitive molecular diagnosis of mitochondrial disorders has been greatly hindered by the tremendous clinical and genetic heterogeneity, the heteroplasmic condition of pathogenic mutations, and the presence of numerous homoplasmic mitochondrial DNA (mtDNA) variations with unknown significance. We used temporal temperature gradient gel electrophoresis (TTGE) to detect heteroplasmic mutations from homoplasmic variations in the whole mitochondrial genome.**

Methods: **We screened 179 unrelated patients by TTGE with use of 32 overlapping primer pairs. Mutations were identified by direct sequencing of the PCR products and confirmed by PCR with allele-specific oligonucleotide or restriction fragment length polymorphism analysis.**

Results: **We detected 71 heteroplasmic and 647 homoplasmic banding patterns. Sequencing of the heteroplasmic fragments identified 68 distinct novel mutations and 132 reported sequence variations and mutations; most of them occurred only once. The deleterious nature of some of the novel mutations was established by analyzing the asymptomatic family members and the biochemical and molecular characteristics of the mutation. When the number of mutations was normalized to the size of the region, the occurrence of mutations was 2.4 times more frequent in the tRNA genes than in the mRNA (protein coding) regions.**

Conclusions: **Screening by TTGE detects low proportions of mutant mtDNA and distinguishes heteroplasmic from homoplasmic variations. Results from comprehensive molecular analysis should be followed up with clinical correlation to establish a guideline for complete mutational analysis of the entire mitochondrial genome and to facilitate the diagnosis of mitochondrial disorders.**

Disorders caused by mutations in mitochondrial DNA (mtDNA)[1] represent a group of heterogeneous diseases. Mitochondria are eukaryotic cytoplasmic organelles where oxidative phosphorylation (OXPHOS) takes place to produce the major energy molecule, ATP. This final step of the energy-producing pathway is mediated by five mitochondrial membrane-bound enzyme complexes that transport electrons from NADH and $FADH_2$ to oxygen, generating a proton gradient that ultimately leads to the production of ATP *(1, 2)*. Only 13 of ~85 protein subunits that make up this complex OXPHOS system are encoded by the mitochondrial genome. The remaining OXPHOS polypeptides and proteins involved in mtDNA replication, transcription, and translation and with other regulatory proteins are all encoded by the nuclear genome. Thus, mitochondrial dysfunction can be caused by mutations in either nuclear or mitochondrial genomes.

Because of the high mutation rate of mtDNA and the large number of nuclear genes involved, molecular diagnosis of mtDNA disorders has been focused on ways to identify mutations in mtDNA. The usual tests screen only for the most frequently found mtDNA mutations: A3243G for mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS); A8344G for myoclonus epilepsy, ragged red fibers (MERRF); T8993G/C for neuropathy, ataxia, retinitis pigmentosa (NARP), and Leigh syndrome; G11778A for Leber hereditary optic neuropathy (LHON); and mtDNA deletions for Kearns–Sayre syndrome *(3–5)*. A recent study combining multiplex PCR and allele-specific oligonucleotide (ASO)

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[1] Nonstandard abbreviations: mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; LHON, Leber hereditary optic neuropathy; ASO, allele-specific oligonucleotide; DGGE, denaturing gradient gel electrophoresis; TTGE, temporal temperature gradient gel electrophoresis; np, nucleotide position; RFLP, restriction fragment length polymorphism; and SNP, single-nucleotide polymorphism.

Exhibit B

analysis to screen 44 known point mutations in 2000 patients with mtDNA disorders demonstrated that only ~6% of the patients had identifiable, disease-causing mutations *(3, 4)*. Because many of the patients had strong clinical, genetic, and pathologic indications of mitochondrial disorders, these data suggest that the majority of pathogenic mutations are yet to be identified. The unidentified mutations may occur in the nuclear genes or anywhere in the mtDNA.

Various indirect mutation detection methods have been developed for rapid screening of unknown mutations in nuclear genes *(6)*. For mtDNA mutations, both psoralen-clamp and GC-clamp have been used for broad-range (0–80% denaturant) denaturing gradient gel electrophoresis (DGGE) *(7, 8)*. Michikawa et al. *(8)* developed the DGGE conditions for 18 psoralen-clamped DNA segments to analyze 20 different known point mutations in tRNA genes. Sternberg et al. *(7)* used the traditional DGGE method with a GC-clamp to investigate 35 unrelated patients with mitochondrial encephalomyopathy. In these studies, the analysis was restricted to tRNA genes only. In addition, the procedures were tedious and the modified primers were expensive. Most of all, the method was not practical to extend to the entire mitochondrial genome for routine screening of mtDNA mutations.

Although direct DNA sequencing has been the gold standard of mutation identification, it is not suitable for mutational analysis of mtDNA because of its inability to detect a low percentage of mutant mtDNA in a heteroplasmic state. However, most of the pathogenic mutations are heteroplasmic. Additionally, direct DNA sequencing is time-consuming, labor-intensive, and cost-ineffective to sequence the entire 16.6-kb mitochondrial genome in all patients suspected of mtDNA disorders. Recently, we established a simple and effective mutation detection method, temporal temperature gradient gel electrophoresis (TTGE), for rapid screening of mtDNA mutations *(9)*. TTGE clearly distinguished heteroplasmic mutations from homoplasmic polymorphisms, and it was sensitive enough to detect heteroplasmic mutations at as low as 4% in DNA fragments as large as 1 kb. In our previous studies, only 11% of the mitochondrial genome was analyzed by TTGE. In this report we extend the TTGE analysis to include the entire mitochondrial genome.

Materials and Methods

Samples used in this study were from patients referred to the Molecular Diagnostics Laboratory at the Institute for Molecular and Human Genetics at Georgetown University Medical Center for molecular diagnosis of mitochondrial disorders. The study protocol (no. 98-033) was approved by the Institutional Review Board at Georgetown University Medical Center. DNA was extracted from peripheral blood lymphocytes according to published procedures and stored at 4 °C *(10)*. The samples were screened for common mtDNA mutations and deletions through routine diagnosis *(3, 4, 11)*. Samples positive for A3243G, T3271C, G3460A, A8344G, T8356C, G8363A, T8993C, T8993G, G11778A, G14459A, T14484C, and deletions were excluded from this study. The 179 patients studied were not specifically selected on the basis of their clinical presentation, age, or muscle biopsy. Some of these patients were simply requesting the DNA analysis to rule out mitochondrial disorder. Because the clinical features of patients with mitochondrial DNA disorders are heterogeneous and may vary from very mild and nonspecific to very severe with specific neuromuscular dysfunction, all patients suspected of mitochondrial disease were included in this study. The purpose was to determine the mtDNA mutations that cover the entire clinical spectrum of mtDNA disease.

The primers used in PCR amplification, the sizes of PCR products, temperature ranges, and ramp rates of the TTGE analysis are listed in Table 1. Each primer, either forward (F) or reverse (R), was ~20 bases long and was named as the nucleotide position (np) number of the mtDNA from the 5′ end of the primer. Each region is named based on the gene or genes it encodes. The tRNA genes are named after the single letter of the amino acid that they carry. Each 25-μL PCR reaction mixture contained 1× Perkin-Elmer PCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.24 μM each primer, 0.75 U of *Taq* DNA polymerase (Applied Biosystems), and 10 ng of total DNA. The reaction mixture was denatured at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, reannealing at 55 °C for 45 s, and extension at 72 °C for 45 s. The PCR reaction was completed by a final extension cycle at 72 °C for 4 min. PCR products were denatured at 95 °C for 30 s and slowly cooled to 45 °C for a period of 45 min at a rate of 1.1 °C/min. The reannealed homo- and heteroduplexes were kept at 4 °C until they were loaded on the gel. TTGE was performed on a Bio-Rad D-Code apparatus. Two back-to-back 20 cm × 20 cm × 1 mm polyacrylamide (acrylamide:bisacrylamide = 37.5:1) gels were prepared in 1.2× Tris-acetate-EDTA buffer containing 9 mol/L urea. A 5-μL portion of the denatured and reannealed PCR products was loaded on the gel. Electrophoresis was carried out at 145 V for 4–5 h at a constant temperature increment of 1–2 °C/h, as shown in Table 1. The temperature range was determined by computer simulation (MacMelt software; Bio-Rad Laboratories). The gels were stained with 2 mg/L ethidium bromide for 5 min and imaged with a digital charge-coupled device gel documentation system. Confirmation of the nucleotide alteration was by direct DNA sequencing of the PCR product with use of a BigDye terminator cycle sequencing reagent set (Perkin-Elmer) and an ABI 377 (Applied Biosystems) automated sequencer. To identify mutations in low-percentage mutant mtDNA, the homoduplex mutant or the heteroduplex bands were excised from the TTGE gel and PCR-amplified before sequence analysis. Once the mutation was

Table 1. TTGE conditions.

Region	Forward primer	Reverse primer	Size, bp	Temperature range, °C	Ramp rate, °C/min	Gel, %
F	mtF467	mtR828	362	53–62	1.8	6
12S	mtF770	mtR1424	655	55–59	1	5
V	mtF1351	mtR1762	412	52–61	1.5–2.0	6
16s.1	mtF1706	mtR2454	749	50–58	1.6	4.5
16s.2	mtF2361	mtR3135	775	53.5–62	1.5	4.5
L	mtF3085	mtR3758	674	54–61	1.2–1.5	5
ND1	mtF3679	mtR4051	373	52–63	1.8	6
IQM	mtF4013	mtR4508	496	52–55.5	1–1.2	5
ND2.1[a]	mtF4444	mtR4917-na	483	52–57	1.1	5
ND2.2[a]	mtF4881	mtR5539-na	668	52–59	1.8	5
WANCY	mtF5460	mtR6016	557	52–58	1.2–1.8	5
COI.1	mtF5960	mtR6656	697	56–64	1.5–1.7	5
COI.2	mtF6606	mtR7282	677	52–59	1.5	5
SD	mtF7234	mtR7921	688	50–58	1.3–1.5	5
K	mtF7804	mtR8380	577	50–55	1.2–1.5	5
ATP6.1[a]	mtF8295	mtR8600	306	53–63	1.8–2	6
ATP6.2[a]	mtF8416	mtR9169	754	52–60	1.5	4.5
COIII	mtF9104	mtR9868	765	56–62	1–1.2	4.5
GR1[a]	mtF9827	mtR10286	460	52–57	1.2	5
GR2[a]	mtF10126	mtR10629	504	50–58	1.6	5
ND4.1	mtF10551	mtR11150	600	52–58	1.5	5
ND4.2	mtF11091	mtR11757	667	53–62	1.8	5
HSL	mtF11688	mtR12360	673	48–57	1.5	5
ND5.1	mtF12239	mtR13007	769	50–60	1.6	4.5
ND5.2	mtF12949	mtR13738	791	52–60	1.2	4.5
ND6	mtF13695	mtR14499	805	55–58	1	4.5
E	mtF14459	mtR15185	727	52–61.5	1.2–1.6	4.5
Cytb	mtF15119	mtR15863	745	52–58	1.5–1.8	4.5
TP	mtF15812	mtR16133	322	50–58	1.6	6
Dloop1	mtF16100	mtR16544	444	53–62	1.8	6
Dloop2	mtF16411	mtR242	402	53–62	1.8	6
Dloop3	mtF159	mtR524	366	53–62	1.8	6

[a] These regions were also analyzed in a single DNA fragment of 1095 bp (mtF4444 and mtR5539), 875 bp (mtF8295 and mtR9169), and 803 bp (mtF9827 and mtR10629) for the ND2, ATP6, and GR regions, respectively.

identified by sequencing, a second method, such as PCR-ASO dot blot or PCR-restriction fragment length polymorphism (RFLP) analysis was used to confirm the mutation and to establish the status of homo- or heteroplasmy *(3, 9)*.

Results

DISTINCTION OF HOMOPLASMIC AND HETEROPLASMIC MUTATIONS BY TTGE

The entire mitochondrial genome was PCR-amplified with 32 pairs of overlapping primers. These 32 DNA fragments varied in size from 306 to 805 bp (Table 1). The amplified fragments totaled 18 953 bp, 14.4% more than the mitochondrial genome of 16 569 bp attributable to the overlapping regions. Because the TTGE conditions varied according to the melting behavior of the DNA fragments, samples were analyzed in batches by regions. The principles, sensitivity, and specificity of TTGE have been discussed previously *(9)*. On TTGE analysis, a single band shift represents a homoplasmic nucleotide substitution (Fig. 1A, lane 1) and a multiple banding pattern represents a heteroplasmic mutation (Fig. 1A, lanes 3–5). DNA fragments showing abnormal banding patterns, particularly multiple bands, on TTGE analysis were sequenced for identification of the exact mutations. In lanes 3 and 4 in Fig. 1A, the two lower bands are the homoduplexes of the wild type and the mutant, whereas the upper two bands are the heteroduplexes. Lane 5 in Fig. 1 is also a sample from a heteroplasmic mutant. However, the two homoduplex bands were not separated. Sequence analysis revealed a homoplasmic mutation of C15904T for lane 1 (see Fig. 1B) and a heteroplasmic mutation of T15852C and a heteroplasmic C15849T for lanes 3 and 4, respectively (see Fig. 1C). The heteroplasmic mutations of G15995A for lane 5 are shown in Fig. 1D. The heteroplasmic states of these mutations were confirmed by ASO dot-blot analysis (data not shown). A DNA fragment containing more than four bands on TTGE analysis suggests the presence of more than one heteroplasmic mutation (see Fig. 2A). Results of sequencing analysis indeed

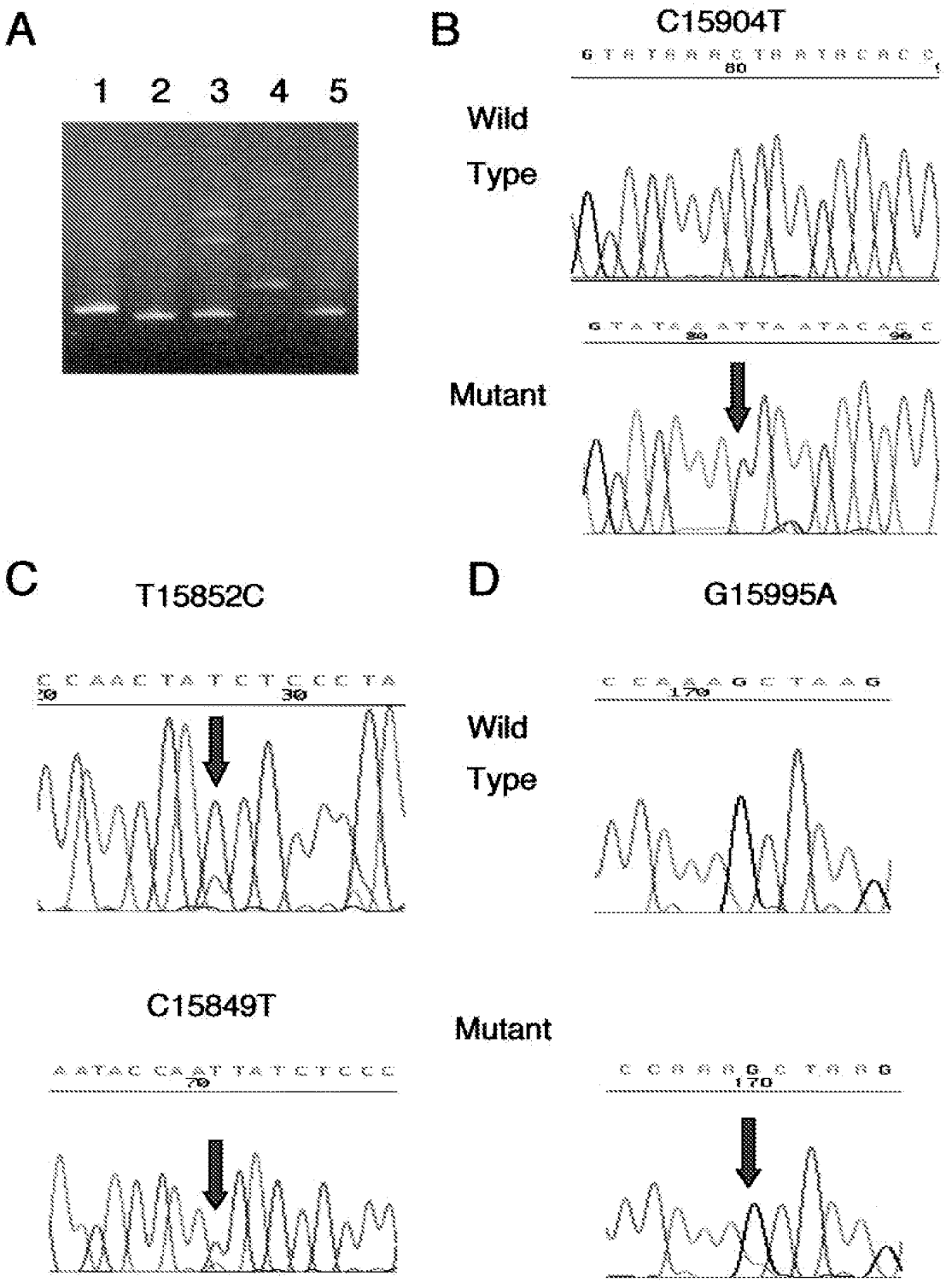

Fig. 1. TTGE analysis of TP region of mtDNA and sequencing results of the mutations.

PCR primers are 15812 and mtR16133. The size of the PCR product is 322 bp. (*A*), *lane 1*, homoplasmic band shift up; *lane 2*, wild type; *lanes 3–5*, heteroplasmic mutations. (*B*), sequencing result of *lane 1*, C15904T. (*C*), sequencing result of *lanes 3* and *4*, T15852C and C15849T, respectively. (*D*), sequencing result of *lane 5*, G15995A.

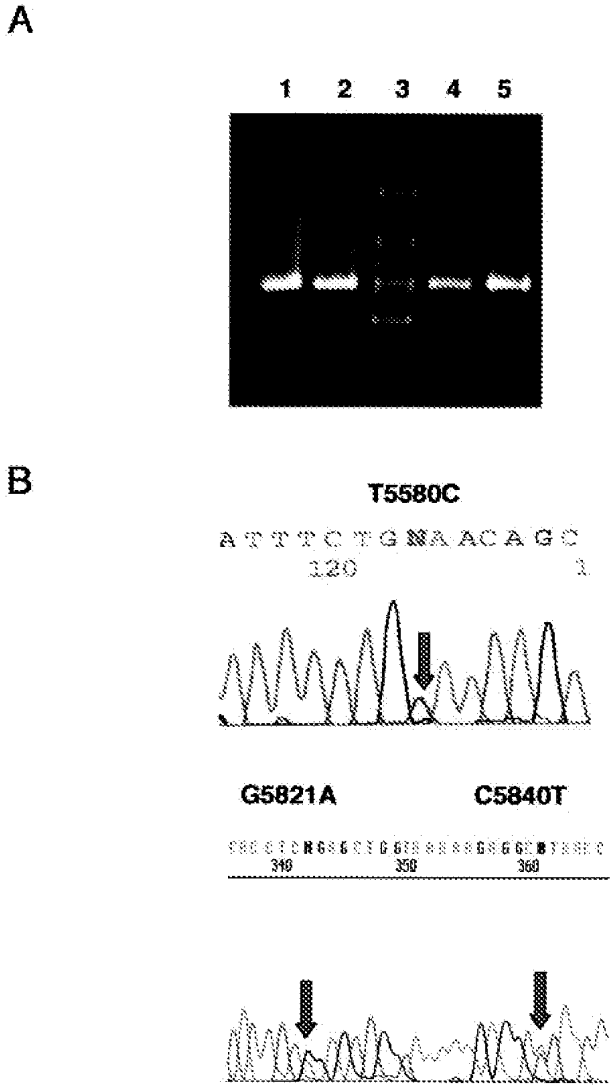

Fig. 2. TTGE analysis of WANCY penta tRNA region.

PCR primers are 5460 and mtR6016. The size of the PCR product is 567 bp. (*A*), *lanes 1*, *2*, *4*, and *5* are wild-type patterns; in *lane 3*, multiple (more than four bands) banding pattern suggests the presence of more than one heteroplasmic mutation. (*B*), sequence results of *lane 3* showing three heteroplasmic mutations, T5580C, G5821A, and C5840T, in the same DNA fragment.

revealed multiple heteroplasmic mutations (T5580C, G5821A, and C5840T) in this DNA fragment (see Fig. 2B).

NUMBER OF mtDNA MUTATIONS AND VARIATIONS DETECTED

TTGE analysis of the entire mitochondrial genome of the 179 patients detected a total of 71 heteroplasmic and 647 homoplasmic banding patterns (Table 2). Fifty-six percent (40 of 71) of the heteroplasmic mutations occurred in the hypervariable D loop and flanking regions (Table 2, np 16100–np 828). In addition, a total of 647 homoplasmic band shifts were detected. Among them, 224 (34.6%) were in the D loop and its flanking region (np 16100–np 828). Because most of the homoplasmic alterations were known benign polymorphisms, they were not followed by sequencing. One of the advantages of TTGE is that it distinguishes heteroplasmic from homoplasmic DNA alterations. Therefore, much time and effort are saved because the enormous number of DNA fragments with homoplasmic DNA polymorphisms do not need to be sequenced. In addition to the D loop, several regions are highly polymorphic, for example, the E, V, and 16S regions. The MITOMAP database *(12)* lists 33 known RFLPs and 16 reported single-nucleotide polymorphisms (SNPs) in the E region *(13)*. In the V region, there are 15 RFLPs and 3 additional SNPs *(13)*. These polymorphisms occur in various frequencies among different ethnic groups. The majority of the homoplasmic variations represent common nucleotide polymorphisms. On the other hand, we expect that most of the heteroplasmic mutations are unique nucleotide changes that occur only once. Naturally, not all heteroplasmic mutations will be clinically important. For example, the heteroplasmic nucleo-

Table 2. Number of hetero- and homoplasmic banding patterns detected by TTGE in the entire mtDNA.

Region	Position, np	No. of heteroplasmic patterns detected	No. of homoplasmic patterns detected
F	467–828	10	27
12S	770–1424	4	4
V	1351–1762	0	30
16s.1	1706–2454	0	45
16s.2	2351–3135	0	11
L	3085–3758	0	17
ND1	3679–4051	0	12
IQM	4013–4508	1	0
ND2.1	4444–4917	0	12
ND2.2	4881–5539	0	2
WANCY	5460–6016	6	8
COI.1	5960–6656	1	11
COI.2	6606–7282	3	21
SD	7234–7921	0	15
K	7804–8380	1	15
ATP6.1	8295–8600	0	3
ATP6.2	8416–9169	1	12
COIII	9104–9868	1	29
GR1	9827–10286	0	24
GR2	10126–10629	0	8
ND4.1	10551–11150	1	18
ND4.2	11091–11757	0	9
HSL	11688–12360	3	1
ND5.1	12239–13086	0	8
ND5.2	12949–13739	1	4
ND6	13695–14499	0	3
E	14459–15185	0	45
Cytb	15119–15863	0	5
TP	15812–16133	6	24
Dloop1	16100–16544	19	59
Dloop2	16411–242	7	92
Dloop3	159–577	4	73
Total		71	647

Table 3. Novel mtDNA mutations found by TTGE and sequencing.

Mutation	No. of occurrences	Gene	Codon	Amino acid change	Homo-/ Heteroplasmy	Confirmed by	Significance
286-291 del	1	D loop				Sequencing	mTF1 binding site
AA					Homo		
A723C	2	12S rRNA			Hetero	Sequencing	
A745G	1	12S rRNA			Hetero	Sequencing	
A1386T	1	12S rRNA			Hetero	Sequencing	
G1442A	1	12S rRNA			Homo	Sequencing	
C1721T	1	16S rRNA			Hetero	RFLP & ASO	
G2098A	1	16S rRNA			Homo	Sequencing	
C3333T	1	ND1	CTC-CTT	L9L	Homo	Sequencing	
T3509C	1	ND1	ATC-ACC	I68T	Homo	Sequencing	
C4029T	1	ND1	ATC-ATT	I241I	Homo	Sequencing	
G4048A	1	ND1	GAC-AAC	D248N	Hetero	RFLP	
A4203G	1	ND1	GCA-GCG	A299A	Hetero	RFLP	
T4363C	1	Q			Homo	ASO	
T4454C	1	M			Hetero	RFLP & ASO	Conserved
C4485A	1	ND2	CAA-GAA	Q6K	Hetero	Sequencing	
C4490G	1	ND2	CCC-CCG	P7P	Hetero	Sequencing	
T4586C	1	ND2	GCT-GCC	A39A	Homo	Sequencing	
C4735A	1	ND2	ACT-AAT	T89N	Homo	Sequencing	
C5462T	1	ND2	GCC-GCT	A331A	Homo	Sequencing	
A5496G	1	ND2	ATA-GTA	M343V	Hetero	Sequencing	
C5499T	1	ND2	CTA-TTA	L344L	Hetero	Sequencing	
A5539G	1	W			Homo	ASO	
T5561C	1	W			Homo	Sequencing	
T5567C	1	W			Homo	Sequencing	
T5580C	2	Noncoding			Hetero	Sequencing	W+4(A-7)
A5584G	1	Noncoding			Homo	ASO	W+8(A-3)
G5585A	1	Noncoding			Homo	ASO	W+9(A-2)
C5840T	2	Y			Hetero	RFLP	
G6446A	1	COI	ACG-ACA	T181T	Homo	Sequencing	
C6473T	1	COI	ATC-ATT	I190I	Homo	Sequencing	
C6713T	1	COI	TAC-TAT	Y270Y	Homo	Sequencing	
A6752G	2	COI	CTA-CTG	L283L	Homo	Sequencing	
T7389C	1	COI	TAT-CAT	Y496H	Homo	Sequencing	
A7729G	1	COII	ACA-ACG	T48T	Homo	Sequencing	
A8039T	1	COII	ATA-TTA	M152L	Hetero	Sequencing	Highly conserved
A8326G	1	K			Homo	Sequencing	

tide changes in the hypervariable D-loop region may be of little clinical significance.

NOVEL mtDNA MUTATIONS AND/OR VARIATIONS AND THEIR DISTRIBUTION IN MITOCHONDRIAL GENOME

Because most pathogenic mtDNA mutations are heteroplasmic, all 71 DNA fragments showing heteroplasmic banding patterns were sequenced. In addition, DNA fragments with unusual band shifts not recognized as common polymorphisms were also sequenced. A total of 201 distinct mtDNA mutations and variations were identified when compared with published sequences (GenBank accession no. J01415) [Refs. *(14–16)* and data not shown]. Among them, 68 were novel and 132 have been reported in the MITOMAP database *(13)*. The heteroplasmic state of mtDNA variations found by TTGE and sequencing was further confirmed by either RFLP or the ASO method, except when the mutant was near-homoplasmic or the heteroplasmic condition was clearly demonstrated by sequencing with both forward and reverse primers. Sixty of the 68 novel mutations occurred only once (Table 3). The remaining occurred two or three times. Of a total of 78 mutations, 50, 16, 7, and 5 were in the mRNA, tRNA, rRNA, and noncoding/D-loop region, respectively (Table 3). Thus, the majority, 64.1% (50 of 78) of the novel mutations were in mRNA genes. When the number of novel mutation was normalized to the size of the gene, the ratio of the density of the novel mutations (variations) per 100 bp was 1:2.41:0.64:0.93 for mRNA:tRNA:rRNA:noncoding/D-loop region, showing a high frequency of mutations at tRNA region (Table 4). C12239T in $tRNA^{ser}$ was detected three times in either the homo- or heteroplasmic state. This probably represents a common variation that is not recorded in the MITOMAP database.

Table 3. Continued

Mutation	No. of occurrences	Gene	Codon	Amino acid change	Homo-/ Heteroplasmy	Confirmed by	Significance
A8566G	2	ATPase 8	CAA-CAG	Q67Q	Homo	Sequencing	
C8655T	1	ATPase 6	ATC-ATT	I43I	Homo	Sequencing	
A8836G	1	ATPase 6	ATG-GTG	M104V	Homo	Sequencing	
C9042T	1	ATPase 6	CAC-CAT	H172H	Homo	Sequencing	
A9347G	1	COIII	CTA-CTG	L47L	Homo	Sequencing	
G9755A	1	COIII	GAG-GAA	E183E	Homo	Sequencing	
C9818T	1	COIII	CAC-CAT	H204H	Homo	Sequencing	
T10007C	1	G			Homo	Sequencing	
T10031C	1	G			Homo	Sequencing	
C10664T	1	ND4L	GTC-GTT	V65V	Homo	Sequencing	
G10686T	1	ND4L	GTG-TTG	V73L	Hetero	Sequencing	
G10688A	1	ND4L	GTG-GTA	V73V	Homo	Sequencing	
G10690T	1	ND4L	GGC-GTC	G74V	Hetero	Sequencing	
T10810C	1	ND4	CTT-CTC	L18L	Homo	Sequencing	
A10876G	1	ND4	CTA-CTG	L39L	Homo	Sequencing	
T10907C	3	ND4	TTT-CTT	F50L	Homo	Sequencing	
A11038G	1	ND4	AAA-AAG	K93K	Homo	Sequencing	
T11732G	1	ND4	TTA-GTA	L325V	Hetero	Sequencing	
A11884C	1	ND4	CTA-CTC	L375L	Hetero	Sequencing	
G12207A	1	S			Hetero	ASO	First base of tRNA absent in mother
C12239T	3	S			Homo/Hetero	RFLP & ASO	
A12273C	1	L(CUN)			Homo	Sequencing	
A12280G	1	L(CUN)			Homo	Sequencing	
T12338C	1	ND5	ATA-ACA	M1T	Hetero	Sequencing	Initiation codon of ND5
A13276G	1	ND5	ATG-GTA	M314V	Homo	Sequencing	
C13506T	1	ND5	TAC-TAT	Y390Y	Homo	Sequencing	
C14284T	1	ND6	GAG-GAT	E111E	Homo	Sequencing	
G15596A	2	CytB	GTC-ATC	V284I	Homo	Sequencing	
A15848G	1	CytB	ACT-GCT	T368A	Hetero	RFLP & ASO	Highly conserved
C15849T	1	CytB	ACT-ATT	T368I	Hetero	ASO	HIghly conserved
T15852C	1	CytB	ATC-ACC	I369T	Hetero	Sequencing	Mildly conserved
G15884C	1	CytB	GCC-CCC	A380P	Hetero	Sequencing	
G15995A	1	P			Hetero	ASO	Absent in mother

A high frequency of heteroplasmic variations was detected in the noncoding region between $tRNA^{trp}$ and $tRNA^{ala}$. They were T5580C, A5584G, and G5585A mutations located at the $tRNA^{trp}$+4, +8, and +9 positions, respectively, corresponding to the $tRNA^{ala}$-7, -3, and -2 positions. Because the processing of polycistronic RNA transcripts depends on the secondary structure and precise cleavage of the precursor, nucleotides between two tRNA genes may be important for structural recognition by the processing enzyme.

Numerous reported mtDNA variations were found by sequencing the heteroplasmic DNA fragments only (results not shown). Of the 132 reported mtDNA variations and polymorphisms, 81 occurred only once. The remaining occurred two or more times with a total of 273 mtDNA variations. The most frequent heteroplasmic mutations were found in the hypervariable D-loop region containing the short tandem repeats at 514–523 $(CA)_n$ and 568–573 $(C)_n$, which occurred 23 and 10 times, respectively, in both hetero- and homoplasmic states of insertion, deletion, or point mutation. These regions are right next to the binding site for mtDNA transcription factor (mtTF1). The T16189C variation occurred very frequently. The T16189 nucleotide is located in the middle of a stretch of 10–13 Cs. Substitution of T with a C produces a continuous stretch of C that is more susceptible to insertion and deletion during DNA replication. This is reflected in the observation that insertion of one to three Cs was accompanied by a T16189C substitution. The G5821A mutation in $tRNA^{cys}$ occurred six times, mostly in heteroplasmic state. This nucleotide is located in the amino acid-carrying stem region. Substitution of G with A disrupts a base pair in the stem that may destabilize the tRNA structure.

Among the mtDNA variations that have been described, A1438G (in 12S rRNA), A3397G (M31V of ND1),

and A15924G ($tRNA^{trp}$) are known to cause disease or to be associated with diabetes mellitus, Alzheimer/Parkinson disease, and lethal infantile mitochondrial myopathy, respectively *(17–20)*. T4216C (Y304H of ND1), G15257A (D171N of cytb), G15812A (V356M of cytb), are secondary, synergistic mutations for LHON. Three mutations, A3397G, T4216C, and A15924G, were found in a heteroplasmic state in our patients. A12308G, a frequent polymorphism present in 18% of healthy controls *(4)*, is reported to be associated with chronic progressive external ophthalmoplegia *(21)*. Two mutations, T7389C [Y469H of cytochrome c oxidase (COXI)] and C8472T (P36L of ATPase 8) were recently reported somatic mutations in papillary thyroid and ovarian carcinomas, respectively. Y469H changes an aromatic amino acid to a basic amino acid, and P36L changes a secondary amino acid to a hydrophobic amino acid; these should be considered as drastic alterations that may have pathogenic consequences.

CLINICAL SIGNIFICANCE OF NOVEL MUTATIONS

Although the clinical significance of most of the novel mutations has not been determined, several of them are believed to be deleterious. For example, the heteroplasmic G15995A mutation disrupts the first base pair of the stem region at the anticodon loop of $tRNA^{pro}$. The patient, a 21-year-old female, was heteroplasmic for the mutation in her blood. Her asymptomatic mother did not have the mutation. The G at np 15995 is highly conserved across species, from the sea urchin to mammals. The fruit fly has an A at np 15995, but it has a T at the corresponding pairing position to form the conserved stem structure. In addition, we did not detect this mutation in 130 healthy controls by the ASO method (Table 5). Clinically, the patient had proximal muscle weakness and increased deep tendon reflexes. She had progressive movement disorders with frequent tremors involving the trunk and face; she also had a history of headaches, stimulus-sensitive myoclonus, and acidosis. In addition to the G15995A mutation, the patient also had a near-homoplasmic A8326G mutation in $tRNA^{Lys}$. Her mother's DNA contained 89% A8326G mutant. The A8326G mutation is located at the anticodon region, adjacent to the first nucleotide of the triplet codon. The A8326 nucleotide is invariant across species, from yeast to humans. In our study, it was not present in 130 healthy control individuals. We believe this mutation is also deleterious because of the high degree of evolutionary conservation. It is not clear whether both mutations contribute to the clinical condition of the patient.

The T12338C mutation changes the initiation codon of methionine in ND5 to threonine. This mutation was present in a nearly homoplasmic state in both blood and muscle specimens from a patient who died on the second day of life with severe lactic acidosis (33 mmol/L). The patient had a history of intrauterine growth retardation, microcephaly, severe metabolic acidosis with an initial pH of 6.7, and a lactate/pyruvate ratio of 100. Autopsy revealed microvesicular steatosis of the liver. Histopathologic studies of the skeletal muscle revealed abnormal mitochondria. Neuropathology findings were consistent with an encephaloclastic process secondary to chronic energy deprivation. The proliferation of vessels in the brain stem and cystic degeneration in the deep gray matter was suggestive of Leigh disease. The patient's clinical findings were clearly indicative of a mitochondrial disorder. However, analysis of the patient's asymptomatic mother revealed the same mutation. Therefore, the T12338C mutation cannot be confirmed as the primary cause of the patient's clinical condition. It is of note that this methionine can be used as initiation site.

The G12207A mutation is located at the first base of $tRNA^{ser}$ (AGY). The patient was a 10-year-old female who had this mutation in both muscle and blood. This patient had a history of developmental delay, impaired growth and feeding disorder, lesions within her basal ganglia, cerebral atrophy, proximal muscle weakness, increased blood lactate (13 mmol/L with a lactate/pyruvate ratio of 31), liver dysfunction, and fatty infiltration of her muscle. The mutation was apparently homoplasmic in the patient, but it was not present in her unaffected mother or 130 healthy controls (Table 5). OXPHOS assays were consistent with complex I deficiency. The muscle biopsy revealed ragged red fibers and pleomorphic mitochondria. Real-time quantitative PCR analysis showed that this patient's mitochondria are proliferated with an mtDNA content 2.4 times higher than normal. All these findings are consistent with a mutation in the tRNA gene of mtDNA.

The patient who carried the heteroplasmic A3397G mtDNA was also heteroplasmic for the T4216C mutation. Both mutations have been known to be disease causing, and both were previously reported to be homoplasmic *(20, 22)*. This patient was a 5-year-old male who presented with seizure, developmental delay, mental retardation, hypotonia, movement disorder, microcephaly, failure to thrive, optical neuropathy, and abnormal magnetic resonance imaging findings. His mother carried neither the T4216C nor the A3397G mutation. The A3397G mutation encodes for the substitution of methionine with valine in a highly conserved region of ND1. It has been reported to be associated with Alzheimer/Parkinson disease. The T4216C mutation changes a tyrosine residue to histidine in the minimally conserved region of ND1. This mutation has been reported to cause synergistic effects on LHON. In addition to A3397G and T4216C, both the patient and his mother carried heteroplasmic A4203G (ND1; no amino acid change) and G4048A (ND1; aspartate to asparagine) mutations. The patient is currently blind. The G4048A (D248N) mutation was recently found as a somatic mutation in papillary carcinomas. We believe the combination of several mutations may be the cause of the patient's disease state.

The G15884C mutation in cytochrome b encodes for the

Table 4. Distribution of mtDNA mutations in mitochondrial genome.

	rRNA	tRNA	mRNA	Noncoding/D loop	Total
Entire genome, bp	2513	1505	11 340	1211	16 569
Percentage of genome	15.2	9.08	68.4	7.3	100
Novel mutations found	7	16	50	5	78
Mutations per 100 bp (mutation density)	0.28	1.06	0.44	0.41	0.47
Ratio of mutation density to mRNA	0.64	2.41	1	0.93	
Reported variations	14	20	88	151	273
Variations per 100 bp	0.56	1.33	0.77	12.47	1.65
Ratio	0.73	1.72	1	16.19	
Total novel and reported mutations	21	35	138	156	352
Mutation density	0.84	2.39	1.22	12.88	2.12
Ratio of mutation density to mRNA	0.69	1.96	1	10.56	

substitution of alanine with proline. A G15884A mutation that encodes for the substitution of alanine with threonine has been reported in the MITOMAP database as a polymorphism. The change of alanine to proline, a secondary amino acid, may have detrimental effects on protein structure and function. However, because this amino acid is at the COOH terminus of cytochrome b, the effect may be minimal. Nevertheless, the patient who harbored this mutation was also homoplasmic for a novel mutation, T4363C, a moderately conserved nucleotide located in the anticodon loop of $tRNA^{gln}$. This patient was a 7-year-old female who presented with developmental delay, gastrointestinal reflux, retinitis pigmentosa, sensorineural hearing loss, and failure to thrive. Studies of the matrilineal family members will be necessary to determine whether one or both of these mutations are contributing to the patient's clinical presentation. The T4363C mutation was not found in 130 healthy controls.

Studies to establish the structural/functional importance and clinical significance of other novel mutations are currently underway. Analysis of asymptomatic family members will be necessary.

FREQUENCY OF mtDNA MUTATIONS IN HEALTHY CONTROLS

One of the criteria for a deleterious mutation is that the mutation should be cosegregated with the disease and not present in healthy controls. We assessed the presence of nine novel and five previously reported mtDNA mutations or variations in 130 healthy controls and 213 patients by the ASO method (Table 4). Statistical analysis revealed no significant difference in the frequencies of G1719A, C1721T, A5539G, A5581G, T10463C, C15904T, A15907G, or G15928A between control and patient groups, suggesting that these are probably benign polymorphisms (five of these were reported as polymorphisms). T4363C, T4454C, G12207A, and G15995A, which occurred only once in 213 patients and not in any of the 130 healthy controls, are likely to be clinically significant as described above. The G5585A mutation occurred six times and the A5584G occurred once in the patient group, but neither occurred in the healthy controls. Whether these nucleotide alterations play a role in disease pathogenesis will require more extensive biochemical and molecular studies. Mutations that occurred in evolutionarily highly conserved

Table 5. Frequency of mutations in control and patient groups.

Nucleotide change	No. of patients (%) n = 213	No. of controls (%) n = 130	Homoplasmy	Heteroplasmy	Comparison of mtDNA sequences across species[a]
G1719A	17 (8%)	9 (6.9%)	−	+	NA[b]
C1721T	3 (1.4%)	5 (3.8%)	−	+	NA
T4363C	1 (0.5%)	0	+	−	Moderately conserved
T4454C	1 (0.5%)	0	+	−	Minimally conserved
A5539G	1 (0.5%)	1 (0.77%)	−	+	Highly conserved
A5584G	1 (0.5%)	0	−	+	NA
G5585A	6 (2.8%)	0	−	+	NA
T10463C	16 (7.5%)	9 (6.9%)	+	−	Moderately conserved
G12207A	1 (0.5%)	0	+	−	Minimally conserved
C15904T	4 (1.9%)	3 (2.3%)	−	+	Moderately conserved
A15907G	2 (0.94%)	1 (0.77%)	−	+	Highly conserved
A15928G	18 (8.5%)	6 (4.6%)	+	−	Minimally conserved
G15995A	1 (0.5%)	0	−	+	Highly conserved

[a] Nucleotide sequences of rRNA or tRNA and amino acid sequences of proteins of nine difference species were compared: yeast, fruit flies, sea urchin, frog, chicken, rat, mouse, bovine, and human.

[b] NA, not available.

regions may be of clinical significance. The biochemical and clinical effects of the novel mutations are currently under investigation.

Discussion

Screening by TTGE method detects low proportions of mutant mtDNA and distinguishes heteroplasmic from homoplasmic

One of the unique features of mtDNA mutations is the state of heteroplasmy. Disease-causing heteroplasmic mutations are usually accompanied by homoplasmic benign polymorphisms. Thus, an effective method for screening mtDNA mutations must not only be capable of distinguishing heteroplasmic from homoplasmic mutations, but also be able to detect low proportions of mutant mtDNA. As described in this and a previous study *(9)*, the TTGE method fits these requirements. The denaturant in TTGE is temperature. There is no need for a GC-clamp or preparation of a chemical denaturant gradient. The method is simple, fast, cost-effective, and has a high throughput. The entire procedure from setting up the gel cassettes to gel imaging can be completed in an 8-h work day. While electrophoresis is taking place, the PCR for the next day's run can be prepared. Two gel plates, each with either 20 or 25 sample lanes, can be run simultaneously in the D code apparatus (Bio-Rad). It must be noted that TTGE differs from temperature gradient gel electrophoresis. In TTGE, a temporal temperature gradient is established. During the electrophoresis, temperature changes vs time. Thus, at any given time the entire gel is at a homogeneous temperature. In contrast, in temperature gradient gel electrophoresis, there is a constant temperature gradient throughout the gel for the whole run period. This temperature gradient does not change vs time. Therefore, temperature modulation in TTGE can be more accurately controlled, thereby providing a broader and more sensitive separation range. A single nucleotide substitution at as low as 4% in as large as 1 kb can be easily detected *(9, 23)*.

Mutations in high-melting domains within or close to primer sites

Similar to the gold standard, DNA sequencing, TTGE has some difficulties. For example, because the PCR product is not GC-clamped, mutations in the high-melting domain may be difficult to separate. Several lines of evidence show that this is probably not a concern. One reason is that mtDNA is GC rich. The DNA fragments analyzed have an average length of 600 bp; thus, stretches of Gs and Cs can serve as internal GC-clamps. In the report by Sternberg et al. *(7)*, an average of 37 GC pairs was added to the primer for the amplification of a DNA fragment of ~225 bp. One fragment of 430 bp, much larger than the others, was amplified by use of a primer attached to a GC-clamp of only 7 bp. This implied that the GC-clamp can be minimized or eliminated if the DNA fragment is large enough to contain internal GC stretches. One concern is that if the mutation occurs within the GC stretch, it will be missed. Our results demonstrate that typically >95% of the mutations are in G/A or T/C transitions and produce a change between two and three hydrogen bonds. This alteration greatly changes the melting behavior of the double-stranded DNA and can be easily detected by TTGE analysis (see discussion below). Another reason that the lack of a GC-clamp in the PCR product probably is not a concern is that analysis of 109 specimens with known homoplasmic or heteroplasmic mtDNA mutations correctly identified each mutation *(9)*. With TTGE, >97% of unknown Hispanic cystic fibrosis mutations were identified *(24)*. In both studies, the detected mutations spanned the entire DNA fragment, including the high-melting domains. These results suggest that a GC-clamp may be spared in TTGE analysis.

Another concern is the detection of point mutations at or very close to the primer sites. If the mutations are at the 3′ end of the primer, elongation will not occur. Therefore, there will be no PCR product if the mutation is homoplasmic *(25)*. It would be scored as normal if the mutation is heteroplasmic. If mutations are at the middle or close to the 5′ end of the primer site, they do not interfere with PCR amplification and would be scored as normal *(26)*. To solve this problem, overlapping primers can be designed such that a mutation at the primer site will be detected in one of the neighboring fragments. As shown in Table 1, each primer pair is overlapped by an average of 73 bp. The average size of the primer is ~18–20 bp. Lastly, TTGE is sensitive enough to detect mutant mtDNA at frequencies as low as 4% *(9)*. It should be noted that the signal intensity of the mutant bands depends on the equilibrium among wild-type/wild-type and mutant/mutant homoduplexes and wild-type/mutant and mutant/wild-type heteroduplexes. The detection limit was studied by mixing known proportions of two polymorphic DNA fragments. We was found that, in general, mutant DNA present in 5% of the total mtDNA was readily detectable (data not shown). To identify the mutation, the heteroduplex or homoduplex band of the mutant mtDNA is excised from the gel and sequenced *(27)*. It would be very difficult to identify <20% of mutant DNA with certainty by direct sequencing *(27)*. Once a mutation is identified, the heteroplasmic state is confirmed by ASO analysis *(9)*.

Rules for band shift on TTGE analysis

More than 120 mutations and SNPs from both nuclear and mitochondrial genes have been detected by TTGE *(23, 27, 28)*. It has been noted that unless multiple mutations are present, an alteration from an AT pair to a GC pair always produces a downward band shift. On the other hand, change from a GC pair to an AT pair always produces an upward band shift. If there is a heteroplasmic change in the background of homoplasmic polymorphisms, the multiple banding patterns shift accordingly *(9)*. Nucleotide changes between G and C and A and T

without involvement of several hydrogen bond changes may be more difficult to detect. However, among the 69 novel mutations detected, one was G/C change and two were A/T changes. Therefore, they are detectable. Analysis of the mutations listed in the MITOMAP database reveals that only 3.5% of the disease-causing mutations are G-to-C or A-to-T changes. In this study, the percentage of G/C or A/T mutations detected was 4.34% (3 of 69), higher than that reported in the MITOMAP database, suggesting that transversion mutations without changes in the number of hydrogen bonds do not escape detection by TTGE.

To ensure that homoplasmic mutations with subtle band shifts are not missed, a mixing experiment was performed. If two DNA fragments that contained a SNP were not clearly separated by TTGE, on mixing the heteroduplexes migrated slower than the homoduplexes because of the mismatch. Any samples showing an ambiguous band shift could be reanalyzed by mixing studies. Forty samples from two regions that showed no band shifts were reanalyzed by mixing the DNA with a known wild-type DNA. None of them showed heteroduplex bands, suggesting that homoplasmic mutations were not present in these samples.

COMPARISON WITH OTHER REPORTED MUTATIONAL ANALYSES OF THE MITOCHONDRIAL GENOME

Because of the tremendous clinical and genetic heterogeneity of mitochondrial disorders, definitive diagnosis of mitochondrial disease has never been easy. In addition, the heteroplasmic condition and the large number of mtDNA variations with unknown effects on disease pathogenesis have hindered the diagnosis. The gold standard, DNA sequencing, cannot resolve these problems, and it cannot detect low percentages of heteroplasmic mtDNA mutations. In addition, sequencing the entire 16.6-kb genome is simply not practical. Two research groups have tried to unravel this dilemma by developing a comprehensive DGGE method for rapid screening of mtDNA mutations *(7, 8)*. These studies focused on the tRNA regions. Michikawa et al. *(8)* used psoralen-tailed primers to analyze a total of 18 segments, ranging from 141 to 421 bp and spanning 3126 bp (<20%) of the mitochondrial genome, for known mutations. Sternberg et al. *(7)* used 15 pairs of GC-clamped primers to analyze tRNA genes in mtDNA from 35 patients with defined mitochondrial encephalopathy or myopathy associated with ragged red or cytochrome c oxidase-negative fibers. The average length of the GC-tail was 37 bp, and the amplified PCR fragments ranged from 132 to 430 bp, with an average of 225 bp. The authors found 46 different sequence variations, 6 of which were in the heteroplasmic state, including the common A3243G and A8344G mutations. Neither of these methods has been used further for the screening of unknown mutations in the entire mitochondrial genome.

In the present study, we sequenced only the heteroplasmic DNA fragments. We found numerous homoplasmic variations in addition to the heteroplasmic mutations. This is because there are many benign polymorphisms in the background. In addition, the low percentages of heteroplasmy detected by TTGE are not revealed by sequencing. The percentage of mutations in tRNA regions from this comprehensive analysis is similar to that reported by Sternberg et al. *(7)*. It should be noted that in the study by Sternberg et al., all 35 patients had relatively defined clinical features of mitochondrial disease and their muscular mtDNA was analyzed. In our study, the patients were not specifically selected other than that they were suspected of a DNA disorder but were negative for common mutations (mtDNA deletions, A3243G, T3271C, G3460A, A8344G, T8356C, G8363A, T8993C, T8993G, G11778A, G14459A, and T14484C). Approximately 78% of the samples were blood samples without histochemical evidence of mitochondrial myopathy. The remaining were muscle biopsies. Most of the patients had unknown disease etiologies with unexplained multisystemic problems and defective energy metabolism without defined diagnosis. Our goal was to establish a strategy for comprehensive mutational analysis and to determine the frequency and clinical correlation of mtDNA mutations for future diagnostic guidelines.

CLINICAL SIGNIFICANCE OF NOVEL MUTATIONS

One of the most difficult tasks in the molecular diagnosis of mtDNA disorders is to determine the biochemical and clinical significance of the sequence variations. Just as not all mtDNA mutations in the homoplasmic state are benign, not all DNA changes in the heteroplasmic state are pathogenic. The DNA alteration must fit the following criteria to be classified as disease-causing deleterious mutations: (*a*) The mutation should not be present in healthy individuals, and it should be cosegregated with the disease in the affected family. If the mutation is in the heteroplasmic state, unaffected matrilineal family members will have little or no mutant mtDNA. The severity of the clinical conditions of the affected individuals is roughly correlated with mutant loads in the affected tissue. (*b*) The mutation must occur in a structurally and functionally important region. For each mutation, the conservation of nucleotide or amino acid sequences across species should be studied. If a mutation in the mRNA produces a frameshift, nonsense mutation, or a nonconserved missense mutation in a conserved region, the mutation is likely to be deleterious. Other, more extensive studies, such as the study of mitochondrial gene expression by analyzing mtRNA processing and translational protein products in cell cultures or transmitochondrial rho cells, may shed light on the pathogenic role of a novel mtDNA mutation.

In conclusion, we present a novel and comprehensive mutation screening method for rapid detection of both homoplasmic and heteroplasmic mutations in the entire

mitochondrial genome. The ability of TTGE to distinguish homoplasmic from heteroplasmic DNA alterations tremendously reduces the number of DNA fragments to be sequenced. Our results suggest that the mutation density in tRNA genes is ~2.41 times that in mRNA genes. Thus, it is possible to institute a stepwise mutation screening procedure by analyzing the tRNA regions first. It is important to determine the pathogenic role of a newly found mutation. Results from the comprehensive molecular analysis should be correlated with clinical presentation such that guidelines can be established to improve the molecular diagnosis of mitochondrial disorders.

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